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PftetQ and pfmdt copy numbers as predictive molecular markers of decreased ex vivo doxycycline susceptibility in imported Plasmodium falciparum malaria

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Abstract

Background: The objective of this study was to evaluate the distribution of a series of independent doxycycline inhibitory concentration 50% (IC_{50}) values to validate the trimodal distribution previously described and to validate the use of the *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* doxycycline susceptibility in *Plasmodium falciparum* malaria.

Methods: Doxycycline IC_{50} values, from 484 isolates obtained at the French National Reference Centre for Imported Malaria (Paris) between January 2006 and December 2010, were analysed for the first time by a Bayesian mixture modelling approach to distinguish the different *in vitro* phenotypic groups by their IC_{50} values. Quantitative real-time polymerase chain reaction was used to evaluate the *pftetQ* and *pfmdt* copy numbers of 89 African *P. falciparum* isolates that were randomly chosen from the phenotypic groups.

Results: The existence of at least three doxycycline phenotypes was demonstrated. The mean doxycycline IC_{50} was significantly higher in the group with a *pftetQ* copy number >1 compared to the group with a *pftetQ* copy number = 1 (33.17 μM *versus* 17.23 μM) and the group with a *pfmdt* copy number >1 (28.28 μM *versus* 16.11 μM). There was a significant difference between the combined low and medium doxycycline IC_{50} group and the high IC_{50} group in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (0% *versus* 20.69%) or *pfmdt* gene (8.33% *versus* 37.93%). In the logistic regression model, the *pfmdt* and *pftetQ* copy numbers >1 (odds ratio = 4.65 and 11.47) were independently associated with the high IC_{50} group.

Conclusions: Copy numbers of *pftetQ* and *pfmdt* are potential predictive molecular markers of decreased susceptibility to doxycycline.

Keywords: Malaria, *Plasmodium falciparum*, Anti-malarial, *In vitro*, Resistance, Molecular marker, Doxycycline

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Background

Daily administration of doxycycline is currently a recommended chemoprophylactic regimen for travellers visiting malaria-endemic areas with high prevalence of chloroquine or multidrug resistance [1]. In addition, the French malaria consensus recommends quinine and doxycycline for the first-line treatment of *Plasmodium falciparum* severe malaria in Asia and South America. In combination with artesunate or quinine, doxycycline remains the recommendation as the second-line treatment of uncomplicated falciparum malaria or for the treatment of severe malaria as a seven-day course [2]; however, its use is limited. Prophylactic failure of doxycycline against *P. falciparum* has been associated with both inadequate doses [3] and poor compliance [4].

Since September 2002, French troops have participated in the peace-keeping operation, Operation Licorne, in the Ivory Coast. Soldiers had been prescribed doxycycline (100 mg) daily for prophylaxis. Many cases of malaria have been reported, but most of these cases are believed to be the result of poor compliance [5,6]. From 2002 to 2006, 1,787 falciparum malaria cases were observed in French soldiers who were expected to take doxycycline. A surge in the number of malaria cases within three weeks after doxycycline prophylaxis discontinuation is often observed after return [7,8]. Therefore, it is recommended that doxycycline be taken for four weeks after returning from an endemic area. However, resistance can also explain failures of prophylactic doxycycline.

The ability to maximize the efficacy and longevity of anti-malarial drugs for malaria control will depend critically on intensive research to identify *in vitro* markers along with ex vivo and *in vivo* surveillance programmes. It is necessary to identify molecular markers that predict doxycycline resistance or decreased susceptibility in order that active surveillance can monitor temporal trends in parasite susceptibility [9]. Although there have been no reported clinical failures for the treatment of falciparum malaria with doxycycline, a Bayesian mixture modelling approach has distinguished three different *in vitro* phenotypic groups: low, medium and high doxycycline IC_{50} values, among 747 *P. falciparum* isolates obtained from 14 African countries over a nine-year period [10]. The sequences of 11 *P. falciparum* genes that are analogous to those involved in bacterial resistance to doxycycline were obtained from 30 isolates from each phenotypic group. The data suggested that the copy numbers of a *tet*Q GTPase family gene, *pftetQ* (PFL1710c), and a metabolic drug transporter gene, *pfmdt* (PFE0825w), were potential molecular markers of decreased *in vitro* susceptibility to doxycycline in African isolates [11].

The objective of this study was first to evaluate the distribution of a new series of independent doxycycline IC_{50} values assessed by another group for goodness of fit with the trimodal compartment model of doxycycline response previously proposed [10] and then to validate the use of the *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to doxycycline. This was performed by assessing the gene copy numbers in *P. falciparum* clinical isolates that were randomly chosen from the phenotypic groups with different doxycycline IC_{50} values.

Methods

Patients and sample collection

Between January 2006 and December 2010, 484 fresh *P. falciparum* isolates were obtained at the French National Reference Centre for Imported Malaria (Paris) from patients hospitalized with malaria after having returned to France. These samples were successfully assessed for doxycycline susceptibility. Ex vivo testing of doxycycline susceptibility was performed as previously described by a standard 42-hour ^{3}H-hypoxanthine uptake inhibition assay [12]. Batches of plates were tested and validated on the chloroquine-susceptible 3D7 strain and the chloroquine-resistant W2 strain.

The drug concentration that inhibited 50% parasite growth (IC_{50}) was calculated with the inhibitory sigmoid Emax model, with estimation of the IC_{50} through non-linear regression using a standard function of the R software (ICEstimator) [13].

Quantification of pftetQ and pfmdt copy numbers

pfmdt (PFE0825w) and *pftetQ* (PFL1710c) copy numbers were estimated by TaqMan real-time PCR (7900HT Fast Real-Time PCR system, Applied Biosystems) relative to the single-copy gene, *pfβtubulin* (PF10_0084). The following oligonucleotide primers and probes were designed using the Primer Express software v2.0 (Applied Biosystems) for use in the polymerase chain reactions (PCRs): 5′-TTATGCAAACATTTCAAGCTTCCT-3′, 5′- ACCC ATTCCATAACTTAGATTTAGATAACC-3′ and 5′-VIC-TAAAAACAAATTTCGACAAAAGGACAGGAGCC-TA MRA-3′ for *pfmdt*, 5′-ACCCCTTTTTTATCTTACGAA AG-3′, 5′-ATGGTTGTACGTTATATCATATGG-3′ and 5′-VIC-AAAAATGTGGCAACAATTCAGACATGTATC A-TAMRA-3′ for *pftetQ* and 5′-TGATGTGCGCAAGT-GATCC-3′, 5′-TCCTTTGTG GACATTCTTCCTC-3′ and 5′-FAM-TAGCACATGCCGTTAAATATCTTCCAT GTCT-TAMRA-3′ for *pfβtubulin* (Eurogentec). Individual PCRs were performed using 1 X TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 250 nM TaqMan probe and 5 μL template DNA in a final volume of 25 μL The reaction mixtures were prepared at 4°C in a 96-well optical reaction plate (Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min and

50 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was assayed in triplicate and analysed with the SDS software 2.2.1 (Applied Biosystems). The PCR efficiencies of all the primer pairs were evaluated on a dilution series of *P. falciparum* 3D7 genomic DNA. The efficiencies were found to be sufficiently close to obviate the need for any correction factor. Therefore, the $2^{-\Delta\Delta Ct}$ method of relative quantification was used and adapted to estimate the number of copies of the *pfmdt* and *pftetQ* genes [14,15] with the formula $\Delta\Delta Ct = (Ct_{pfmdt} - Ct_{pf\beta tubulin})_{\text{sample}} - (Ct_{pfmdt} - Ct_{pf\beta tubulin})_{\text{calibrator}}$. Genomic DNA extracted from 3D7 *P. falciparum*, which has a single copy of each gene, was used for calibration, whereas *pfβtubulin* served as the control housekeeping gene in all the experiments.

Genetic diversity of Plasmodium falciparum isolates with pftetQ and pfmdt multicopies

Mixed infection could influence read-out in Taqman real-time PCR, potentially leading to false positive results of gene copy number. The genomic DNA of *P. falciparum* isolates with at least two copies of *pfmdt* or *pftetQ* were investigated for genetic diversity at highly polymorphic loci, merozoite surface proteins 1 and 2 (MSP1 and MSP2). The *msp1* and *msp2* loci were genotyped using the nested PCR strategy and conditions previously described [16].

Statistical analysis

The statistical analysis has been designed to answer the specific question of whether *P. falciparum* has different doxycycline susceptibility phenotypes. A heterogeneous population of IC_{50} values was observed; therefore, the data were assumed to represent a univariate Gaussian mixture with k components. Each observation was assumed to originate from one of the k components, and the label of the group from which each observation arose was unknown. The unknowns of the model were the number of components, the means, variances and weights of the different components, and the vector of allocations of the observations. The analysis was performed in two steps. First, reversible jump Monte Carlo Markov Chains (RJMCMC) [17] samplers were used to choose a suitable number of components k, and the present algorithm followed the recommendations of Cappé et al. [18]. After a relevant number of components was chosen, standard Gibbs samplers were run to obtain estimates of the model parameters and to classify the observations [19]. Because of the'label-switching' problem, due to the symmetry in the likelihood of the model parameters, the mixture components should be labelled before making an inference on the parameters [20]. The classical ordering constraint, which was biologically relevant here, was used. The algorithms were run for 5,000 burn-in iterations and 20,000 post-burn-in iterations. These numbers were assumed to be sufficient to obtain reliable results. Moreover, each algorithm was run three times to check that the results between two different runs were similar and that there was no convergence problem [17].

The data were analysed using the R software® (version 2.10.1). The differences in the *pfmdt* and *pftetQ* copy numbers between the phenotypic groups were tested using the Mann Whitney test and the Kruskal-Wallis test. The genotype proportions were compared using the Fisher exact test. The risk of the high doxycycline IC_{50} was analysed using a logistic regression model (univariate and multivariate analysis).

Ethics

Informed consent was not required for this study because the sampling procedures and testing are part of the French national recommendations for the care and surveillance of malaria.

Results

The doxycycline IC_{50} values ranged from 0.49 to 65.1 μM. The mean was 11.64 μM (95% confidence interval, 10.96-12.33). The average parameter estimates for the IC_{50} values by year are given in Table 1.

The triple normal distribution model is represented in Figure 1. The parameter estimates for the three-component mixture model, including the number of isolates in each normal distribution, the mean of the IC_{50} values and the standard deviation for each distribution, are summarised in Table 2. A double normal distribution model and a quadruple normal distribution model were also fitted to the data to assess the validity of considering a three-component mixture (data not shown). These two models fit the data worse than the triple normal distribution model.

Eighty-nine *P. falciparum* isolates (30, 30 and 29) were randomly chosen from the three phenotypic groups, A, B and C, that differed in their doxycycline IC_{50} values. These isolates were classified as follows: low doxycycline IC_{50} group from component A [mean, 4.33 μM (95% CI, 3.39-4.37 μM)], medium doxycycline IC_{50} group from component B [mean, 16.97 μM (95% CI, 16.45-17.49 μM)]

Table 1 Statistical analysis of the 484 doxycycline IC_{50} values by year

Year	IC_{50} number	Mean (μM)	95% CI	IC_{50} min	IC_{50} max
2006	119	10.05	9.14-10.96	0.63	43.7
2007	172	11.91	10.72-13.11	2.34	44.8
2008	59	9.45	8.56-10.35	4.27	23.1
2009	40	12.21	8.01-16.41	0.49	65.1
2010	94	14.3	12.63-15.97	4.55	46.2
Total	484	11.64	10.96-12.33	0.49	65.1

95% CI: 95% confidence interval.

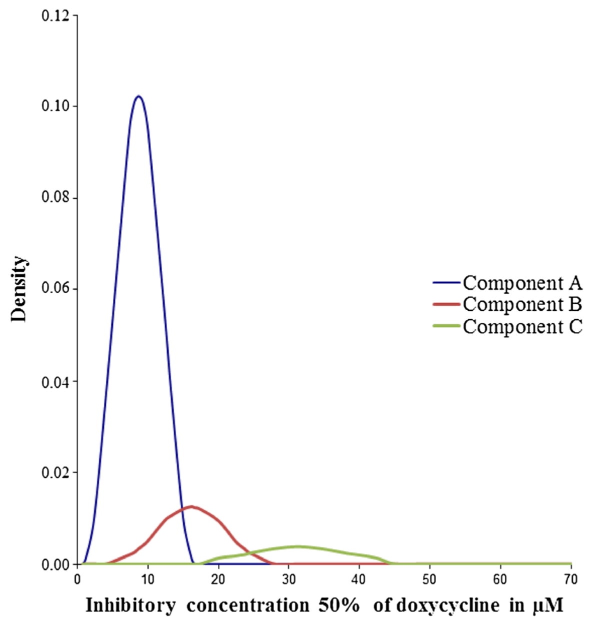

Figure 1 Distribution of the doxycycline IC_{50} values of the 484 *Plasmodium falciparum* isolates (2006 to 2010) in the three-component mixture model (Bayesian mixture modelling approach). The dotted lines represent the three fitted mixtures.

and high doxycycline IC_{50} group from component C [mean, 34.60 µM (95% CI, 31.30-37.90 µM)].

Only one or two copies of *pfmdt* and *pftetQ* were identified in the 89 isolates. All of the isolates with two copies *pfmdt* or *pftetQ* had 1 allelic family for each of the two genes (*msp1* and *msp2*), confirming that these infections were single and not mixed. The mean doxycycline IC_{50} was significantly higher in the group with a *pftetQ* copy number >1 compared to the group with a *pftetQ* copy number = 1 (33.17 µM *versus* 17.23 µM; $P = 0.0041$, Mann Whitney test) (Table 3). The mean doxycycline IC_{50} was significantly higher in the group with a *pfmdt* copy number >1 (28.28 µM *versus* 16.11 µM; $P = 0.0025$, Mann–Whitney test).

The number of *pftetQ* copies was significantly higher in the high doxycycline IC_{50} group compared to the low and medium doxycycline IC_{50} groups (1.21 *versus* 1.0 and 1.0; $P = 0.0014$, Kruskal-Wallis test). The number of *pfmdt* copies was significantly higher in the high doxycycline IC_{50} group compared to the low and medium doxycycline IC_{50} groups (1.38 *versus* 1.13 and 1.03, respectively; $P = 0.0019$, Kruskal-Wallis test).

There was no significant difference between the low and medium doxycycline IC_{50} groups for the *pfmdt* and *pftetQ* copy numbers. Therefore, these two phenotypic groups were combined. There was a statistically significant difference between the low and medium doxycycline IC_{50} combined group and the high doxycycline group in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (0% *versus* 20.69%; $P = 0.0008$, Fisher's exact test) or *pfmdt* gene (8.33% *versus* 37.93%; $P = 0.0021$, Fisher's exact test) (Table 4).

In the logistic regression model (Table 5), the *pfmdt* copy number >1 (adjusted OR = 4.65 [1.31-16.51], $P = 0.0176$) and *pftetQ* copy number >1 (adjusted OR = 11.47 [1.23-106.98], $P = 0.0322$) were independently associated with the high IC_{50} phenotypic group.

Table 2 Parameter estimates for the three-component mixture model for the 484 *Plasmodium falciparum* isolates

Component	Isolates number	Proportion (%)	IC_{50} mean (µM)	Standard deviation
A	393	81.1	8.70	2.87
B	60	12.4	16.18	4.86
C	31	6.5	31.23	10.32

Table 3 Statistical analysis of the doxycycline IC_{50} values based on the *pftetQ* and *pfmdt* copy numbers in 89 *Plasmodium falciparum* isolates

	pftetQ copy number		*pfmdt* copy number	
	= 1	> 1	= 1	> 1
Number of values	83	6	73	16
IC_{50} mean (µM)	17.23	33.17	16.11	28.28
Standard deviation	13.32	6.85	12.53	14.03
95% Confidence interval	14.32-20.13	25.98-40.37	13.19-19.04	20.81-35.76
Minimal IC_{50}	0.49	25.16	0.49	4.62
Maximal IC_{50}	65.11	43.7	44.82	65.11

Discussion

Most prophylactic failures of doxycycline against *P. falciparum* are associated with the use of standard doses resulting in lower than expected serum drug levels [21], inadequate low doses [3], or poor compliance [4,22]. Moreover, doxycycline pharmacokinetic parameters could explain some of these cases. Doxycycline has a short elimination half-life (16 hours) compared to proguanil (24 hours), atovaquone (31–73 hours), chloroquine (two to

Table 4 Statistical analysis of *pftetQ* and *pfmdt* copy numbers in 89 *Plasmodium falciparum* isolates (Fisher's exact test)

	PftetQ		*Pfmdt*	
	Low and medium IC_{50}	High IC_{50}	Low and medium IC_{50}	High IC_{50}
Copy number >1	0	6	5	11
Copy number = 1	60	23	55	18
%	0.00	20.69	8.33	37.93
Fisher's exact test	*P* value	0.0008	*P* value	0.0021

Table 5 Multivariate regression model

Molecular marker	Doxycycline IC_{50} group, number: Low or medium	High	Crude OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
pftetQ copy number						
1	60	23	1.00 (reference)		1.00 (reference)	
> 1	0	6	18.77 (2.18-161.43)	0.0076	11.47 (1.23-106.98)	0.0322
pfmdt copy number						
1	55	5	1.00 (reference)		1.00 (reference)	
> 1	18	11	6.72 (2.06-21.96)	0.0016	4.65 (1.31-16.51)	0.0176

three days), or mefloquine (six to 41 days), and a short mean residence time (63% of the administered dose is eliminated in 27 hours) [8]. In addition, its slow action *in vitro* has a delayed effect upon growth and requires the prolonged incubation of parasites [23]. Determination of the IC_{50} after two generations of parasite growth decreases the 42-hour IC_{50} from ten- to 20-fold [24,25]. However, in practice, the standard 42-hour test remains the method of monitoring doxycycline ex vivo susceptibility.

Maximizing the efficacy and longevity of anti-malarial drugs to control malaria will critically depend on intensive research to identify *in vitro* markers along with the implementation of ex vivo and *in vivo* surveillance programmes, such as those championed by the WorldWide Antimalarial Resistance Network [26]. Therefore, there is a need to identify molecular markers that predict doxycycline resistance, which can provide an active surveillance method to monitor temporal trends in parasite susceptibility [9]. In addition, the early detection of resistance or decreased susceptibility to doxycycline will require that the baseline parasite chemosusceptibility of current isolates from endemic regions is established.

To validate the trimodal distribution model of doxycycline IC_{50} values previously described for *P. falciparum* African isolates [10], the distribution of a new series of independent doxycycline IC_{50} values that were assessed by a separate group under the same technical conditions [12] was evaluated. This analysis was performed with a Bayesian mixture modelling approach. Again, the demonstration of the existence of at least three doxycycline phenotypes was confirmed. All 484 values were classified into three components: component A (IC_{50} mean 8.7 μM), component B (IC_{50} mean 16.2 μM), and component C (IC_{50} mean 31.2 μM). This trimodal distribution model of doxycycline IC_{50} values from imported *P. falciparum* isolates obtained from 2006 to 2010 confirms the previous data [10]. However, the level of the IC_{50} value in each component is different between the series of imported *P. falciparum* isolates obtained from 2006 to 2010 and those obtained from 1999 to 2006 [10]. The IC_{50} doxycycline values from the isolates obtained from 1999 to 2006 were classified into three components: component A (IC_{50} mean 4.9 μM), component B (IC_{50} mean 7.7 μM), and component C (IC_{50} mean 17.9 μM). It appears that components A and B (IC_{50} means 4.9 μM and 7.7 μM, respectively) for the values obtained from 1999 to 2006 have merged into a single component A (IC_{50} mean 8.7 μM) for the values obtained from 2006 to 2010. In addition, the percentage of isolates (78%) in the two components, A and B, for the values obtained from 1999 to 2006 is similar to the percentage of isolates (81%) for component A for the values obtained from 2006 to 2010. The component B (IC_{50} mean 16.2 μM) values obtained from 2006 to 2010 correspond to the component C (IC_{50} mean 17.9 μM) values obtained from 1999 to 2006. A new component, component C (IC_{50} mean 31.2 μM, proportion 6.4%), emerged for the values obtained from 2006 to 2010. These data suggest the emergence of strains with decreased susceptibility to doxycycline. In addition, based on the previously defined cut-off for reduced susceptibility to doxycycline (35 μM) [10], 1.2% of the 747 *P. falciparum* isolates tested from 1999 to 2006 were considered to have decreased susceptibility to doxycycline *versus* 2.7% for the 484 isolates tested from 2006 to 2010.

Plasmodium falciparum possesses a *tetQ* GTPase family gene analogue of the genes that encode bacterial ribosomal protection proteins. These genes are the *pftetQ*, which is involved in bacterial resistance to the cycline drugs, and a multidrug transporter gene, *pfmdt*, which shares a high sequence identity with efflux pumps. In a multivariate logistic regression model, an increased *pfmdt* copy number was associated with high doxycycline IC_{50} values with an adjusted odds ratio (OR) of 7.09 ($p = 0.011$), and an increased *pftetQ* copy number was associated with an adjusted OR of 5.23 ($p = 0.042$) [11]. To validate the use of the *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to doxycycline by assessing the gene copy numbers, 89 (30, 30 and 29) *P. falciparum* clinical isolates were randomly chosen from the three phenotypic groups (A, B and C) with different doxycycline IC_{50} values. These isolates were classified as follows: low doxycycline IC_{50} group from component A [mean, 4.33 μM (95% CI, 3.39-4.37 μM)], medium doxycycline IC_{50} group from component B [mean, 16.97 μM (95% CI, 16.45-17.49 μM)] and high doxycycline IC_{50} group from component C [mean, 34.60 μM (95% CI, 31.30-37.90 μM)]. These isolates were obtained from patients hospitalized with malaria after travel in Cameroon (n = 18), Ivory Coast (n = 14), Mali (n = 11), Niger (n = 5), Burundi (n = 4), Burkina Faso (n = 4), Djibouti (n = 4), Madagascar (n = 4), Congo (n = 5), Ghana (n = 3), Sudan (n = 2), Central African Republic (n = 2), Zambia (n = 1), Rwanda (n = 1), Togo (n = 1), Guinea (n = 1), and nine from Africa without specificity regarding the country.

The mean doxycycline IC_{50} value is significantly higher in the groups with *pftetQ* or *pfmdt* copy numbers >1, suggesting that *pftetQ* and *pfmdt* could be involved in the reduced susceptibility to doxycycline.

The number of *pftetQ* and *pfmdt* gene copies is significantly higher in the high doxycycline IC_{50} group than the low and medium doxycycline IC_{50} groups. However, there is no significant difference between the low and the medium doxycycline IC_{50} groups for the *pfmdt* and *pftetQ* copy numbers. These two phenotypic groups were, therefore, combined. There is a statistically significant difference between the low and medium doxycycline IC_{50} combined group and the high doxycycline group in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (0% *versus* 20.69%; $P = 0.0008$) or *pfmdt* gene (8.33% *versus* 37.93%; $P = 0.0021$). In addition, in the multivariate logistic regression model, an increased *pfmdt* copy number is associated with high doxycycline IC_{50} values with an adjusted OR of 4.65 ($P = 0.0176$), and an increased *pftetQ* copy number is associated with an adjusted OR of 11.47 ($P = 0.0322$). These results are consistent with previous data [11] and confirm the potential use of *pftetQ* and *pfmdt* as predictive molecular markers for decreased *P. falciparum* susceptibility to doxycycline in Africa.

In a study on fresh *P. falciparum* clinical isolates from Dakar, Senegal, it was shown that there was no statistically significant difference between a group with a doxycycline IC_{50} <25 μM and a group with an IC_{50} >25 μM in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (p = 0.079) or *pfmdt* gene (p = 0.066) [27]. However, the significance levels of these associations were just above the *P* value threshold (0.05). It seems that the number of isolates from the high doxycycline IC_{50} group (15.9%) was most likely too low to obtain statistically significant differences, indicating the necessity of assessing the gene copy numbers with more isolates. Another possibility is that over-expression of *pftetQ* or *pfmdt* could confer *in vitro* reduced susceptibility to doxycycline in association with other contributing determinants, which could modulate the *in vitro* response to doxycycline.

In summary, this study demonstrates that copy numbers of the *pftetQ* and *pfmdt* genes are potential predictive molecular markers of decreased *P. falciparum* susceptibility to doxycycline in Africa. Epidemiological studies using large numbers of parasites with reduced susceptibility to doxycycline are now required to determine whether *pftetQ* and *pfmdt* can be used as markers of reduced *in vitro* doxycycline susceptibility.

Competing interests
The authors have declared that they have no competing interests.

Authors' contributions
TG, NW, ML and AP carried out the molecular genetic studies. SH, VH and JLB carried out the ex vivo evaluation of doxycycline susceptibility. The French National Reference Centre for Imported Malaria Study Group supervised, carried out and coordinated the field collections of patient isolates. BP and SB conceived and coordinated the study. SB, MB, CT and BP analysed the data. TG, SB, SH, MB, JLB and BP drafted the manuscript. All the authors read and approved the final manuscript.

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